Immunomodulatory approaches to the treatment of infections

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Review article

The immune system offers a wide variety of potential targets for modulation in the treatment of infectious diseases. Available possibilities permit both prophylactic and therapeutic immunomodulation for supportive and direct treatment of infections. In addition to regular moderate exercise, an adequate diet containing plant polyphenols, particularly from fruit, and micronutrients such as selenium and zinc helps maintain effective host defence against mild to moderate infections. Supplementation with these dietary constituents is usually necessary in critically ill patients and like some standardized phytopharmaceuticals, may also reduce the severity and/or duration of upper airways infections. Administration of probiotics also promotes gastrointestinal immune defence. Several antibiotics, especially the macrolides and tetracyclines, possess in addition to their antibacterial actions, immunomodulatory and/or anti-inflammatory properties which facilitate bacterial killing and/or protect tissue from bystander injury. They may also be useful in prevention of the virally-induced cytokine storm. Immunoglobulins, several recombinant human cytokines and selective agonists of Toll-like receptors are effective against many infections in their own right because of highly specific receptor-mediated actions on defined immune processes. The development of novel receptor-targeted immunomodulators is likely to replace the use of non-specific modulators of innate immunity in promoting host defence during infection.

**Imunomodulacijski pristupi u liječenju infekcija**

Pregledni članak

Imunološki sustav nudi široki izbor potencijalnih ciljnih mjesta za modulaciju kod liječenja infektivnih bolesti. Raspoložive mogućnosti dozvoljavaju i profilaktičku i terapijsku imunomodulaciju za suportivno i direkto liječenje infekcija. Odgovarajuća prehrana koja sadrži biljne polifenole, osobito one iz voća, te mikronutrijente kao što su selen i cink, uz redovitu umjerenu fizičku aktivnost pomaže u održavanju učinkovite obrane domaćina od blagih do umjereno teških infekcija. Ovakva nadopuna prehrani obično je prijeko potrebna kod životno ugroženih bolesnika, tako neki biljni pripravci mogu smanjiti težinu i/ili duljinu trajanja infekcija gornjih dišnih putova. Primjena probiotika također potpomaže imunološkoj obrani gastrointestinalnog trakta. Nekoliko antibiotika, prvenstveno makrolidi i tetraciklini, osim antibakterijskog djelovanja imaju i imunomodulatorna i/ili protuupalna svojstva koja potpomažu uništavanju bakterija i/ili štite tkivo od "bystander" ozljeda. Također, mogu biti korisni i u prevenciji tzv. "citokinske oluje" izazvane virusima. Imunoglobulini, nekoliko rekombinantnih humanih citokina te različiti agonisti receptora sličnih Tollu zbog svog visoko specifičnog djelovanja na određene imunološke procese, posredovanog receptorima učinkoviti su kod mnogih infekcija. Vrlo je vjerojatno da bi razvoj novih imunomodulatora koji ciljaju specifične receptore mogao zamijeniti primjenu nespecifičnih modulatora prirođene imunosti kao pomoći pri obrani domaćina od infekcije.

Targets for immunomodulators in infection

The immune response to infection is very diverse, involving non-specific innate immune reactions to common components of pathogens as well as adaptive immune responses to specific pathogenic antigens. As a consequence, multiple potential targets exist for the modulation of the immune system (Fig 1). The most effective protec-
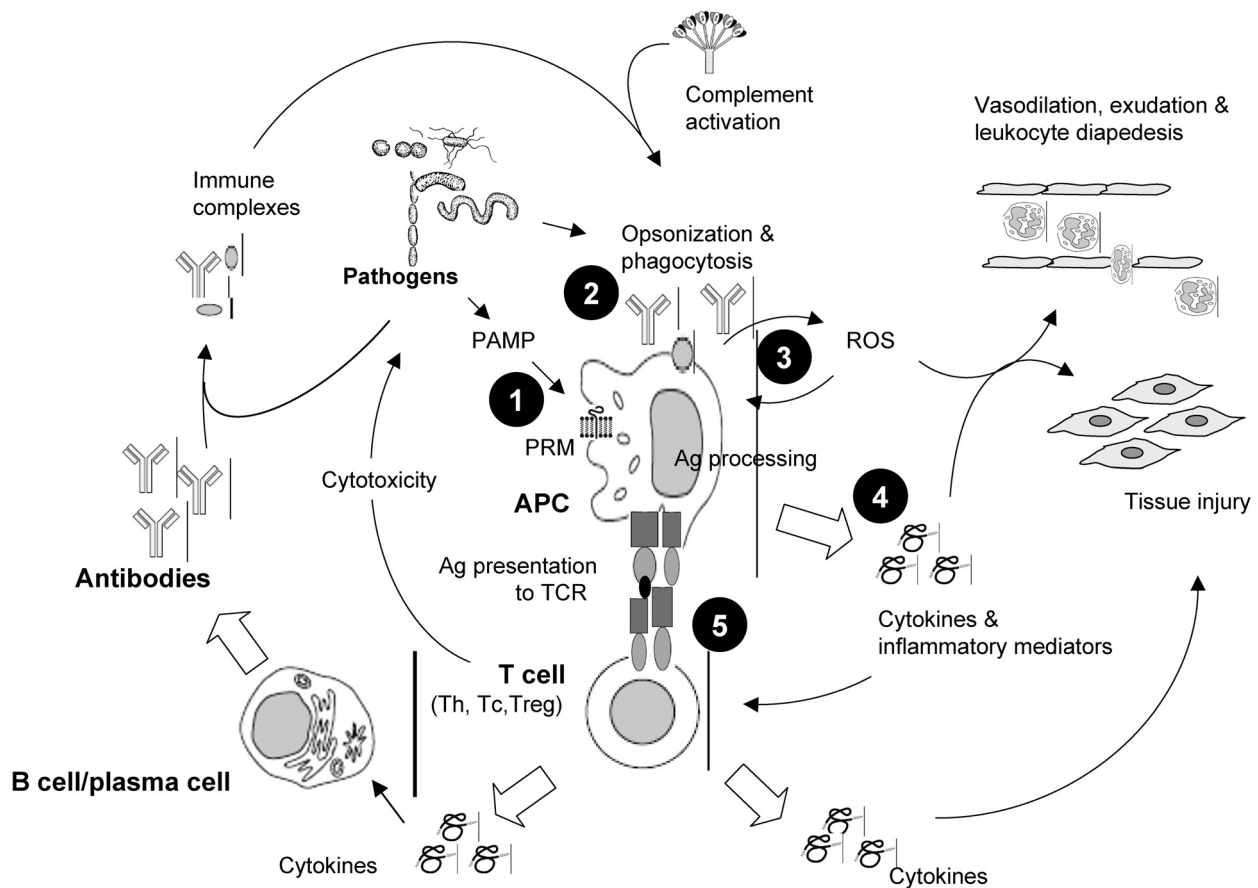
Figure 1. Multiple potential targets for therapeutic modulation of the innate and adaptive immune responses to pathogens. Many of the immunomodulatory approaches to anti-infective prophylaxis and therapy are directed at the interaction between the innate and adaptive arms of the immune system. **1.** Micro-organisms contain broad pathogen-associated molecular patterns (PAMPs) which stimulate cells of the innate immune system by actions on pattern response receptors (PRRs), such as Toll-like receptors (TLRs). These receptors can be stimulated by probiotics, other microbial products and specific TLR agonists so that the cells produce a cytokine pattern (4) which redirects the T cell response to antigen. **2.** Immunoglobulins are the classic immunomodulators as they neutralise and opsonise microbes for phagocytosis and are also able to modulate Fc receptor-mediated responses of phagocytes. The phagocytosis and killing of bacteria can also be modulated by dietary constituents including selenium and zinc and by several different types of phytopharmaceutical and antibacterial drugs. The activity of antigen-presenting cells (APCs) is also specifically promoted by interferons acting as immunomodulatory drugs. **3.** Dietary antioxidants, micronutrients, phytopharmaceuticals and several antibacterial drugs modify the production by phagocytes of reactive oxygen species (ROS). Stimulatory actions can promote bacterial killing, while scavenging actions reduce bystander tissue injury and immunosuppression caused by autocytolysis of immune cells by ROS. **4.** Most immunomodulatory agents are able to modulate inflammatory cytokine production and some antibacterial drugs inhibit inflammatory enzyme production with beneficial effects on bystander tissue injury and inflammation. **5.** Specific modulation of cytokine production, such as that caused by TLR agonists and interferons, results in a change in the type of T cell activated by APCs. Resveratrol, zinc, some probiotics and selected antibacterial drugs are also able to modify T cell responses and thereby antibody production and/or cytotoxicity of T cells to virus-infected cells. **6.** The granulocyte – (G-CSF) and granulocyte/macrophage – (GM-CSF) colony stimulating factors (CSFs) represent specialised immunostimulators which promote the development of new leukocytes from precursors in the bone marrow.

Slika 1. Brojna potencijalna ciljna mjesta za terapijsku modulaciju odgovora prirođene i stečene imunosti na patogene. Mnogi imunomodulacijski pristupi u profilaksi i terapiji infektivnih bolesti usmjereni su na interakciju između prirođene i stečene imunosti – dva kraka imunološkog sustava. **1.** Mikroorganizmi sadrže brojne molekularne obrasce tipične za mikroorganizme (engl. PAMPs) koji stimuliraju stanice prirođene imunosti preko receptora koji prepoznaju te obrasce (engl. PRRs), kao što su npr. receptori slični Tollu (engl. TLRs). Probiotici, neki drugi produkti mikroorganizama kao i specifični agonisti TLR-a mogu stimulirati ove receptore tako da stanice počnu producirati određene citokine (4) koji pak dalje preusmjeravaju T-stanični odgovor prema antigenu. **2.** Imunoglobulini su klasični imunomodulatori budući da oni neutraliziraju i opsoniziraju mikroorganizme za fagocitozu, a također mogu modulirati odgovor fagocita posredovan preko Fc receptora. Fagocitoza i ubijanje bakterija također može biti modulirano i dijetetskim sastojcima koji sadrže selen i cink, kao i pomoću nekoliko različitih vrsta biljnih pripravaka, te antibakterijskih lijekova. Interferoni se mogu ponašati kao imunomodulatorni lijekovi koji specifično potiču aktivnost antigen-prezentirajućih stanica (engl. APCs). **3.** Antioksidansi iz prehrane, mikronutrijenti, biljni pripravci i nekoliko antibakterijskih lijekova modificiraju produkciju reaktivnih molekula kisika (engl. ROS) od strane fagocita.

Djelovanje u smjeru stimulacije produkcije rezultira smrću bakterije, dok djelovanje u smjeru uklanjanja tih reaktivnih molekula reducira "bystander" ozljedu tkiva i imunosupresiju kao posljedicu autocitolize imunih stanica uzrokovane ROS-om. **4.** Većina je imunomodulatornih agenasa sposobna modulirati produkciju proinflamatornih citokina i neki antibakterijski lijekovi inhibiraju produkciju upalnih enzima što ima povoljne učinke na "bystander" ozljedu tkiva i upalu. **5.** Specifična modulacija proizvodnje citokina, kao što je ona uzrokovana agonistima TLR-a i interferonima, rezultira promjenom tipa T-stanica koje aktiviraju APC. Resveratrol, cink, neki probiotici i određeni antibakterijski lijekovi također mogu modificirati T-stanični odgovor, a time i produkciju antitijela i/ili citotoksičnost T stanica prema stanicama inficiranim virusom. **6.** Faktori stimulacije granulocitnih (G-CSF) i granulocitno/makrofagnih (GM-CSF) kolonija predstavljaju specijalizirane imunomodulatore koji potiču razvoj novih leukocita iz prekursorskih stanica u koštanoj srži.

tion is provided by the adaptive immune response which immunizes the host to a specific pathogenic antigen, thereby preventing any further infection by the micro-organism. For an already existing infection, therapy with antibiotics usely springs to mind. The treatment of infection, though, does not only require direct attack on the pathogenic organism. Endogenous host defence reactions can also be modified therapeutically to promote pathogen killing in a manner which avoids or reduces host injury.

The components of the initial innate immune or inflammatory response, which marshal host defences into position and attract leukocytes to the site of infection, are determined to a marked degree by pathogen-associated molecular patterns (PAMPs), including nucleic acids, polysaccharides, and other components. These interact with specific pattern recognition receptors (PRRs), which comprise surface and cytoplasmic receptors on leukocytes. Examples include the Toll-like receptors (TLRs), a family of surface and intracellular receptors, each of which binds a particular class of pathogenic molecules (Table 1). Viruses frequently circumvent detection by TLRs and other PRRs by producing inhibitors of these receptors.

A variety of natural products are able to facilitate defence reactions in a non-specific manner. However, in view of the relatively indiscriminate initial inflammatory response they generate to pathogens, any therapeutic measure which is intended to promote innate immunity also carries the risk of enhancing bystander injury to the surrounding host tissue. In this respect, some antibacterials provide additional benefit to their actions on bacteria by dampening the innate inflammatory response, thereby reducing host injury and potential mortality. Dietary supplementation, particularly with high antioxidant-containing foods also modulates inflammation and there is increasing evidence that probiotic bacteria can promote innate immune defence. Such dietary influences can also be used in a prophylactic approach to common respiratory infections.

Importantly, innate immune responses are also able to modify the specific adaptive immune response by regulating antigen presentation to and activation of T lymphocytes. Among factors which determine the outcome of the activation process, the type of TLR stimulated on an antigen-presenting cell, such as the dendritic cell (DC), is crucial. This can redirect the process towards a T helper-1 (Th1) response against intracellular pathogens, a Th2 response towards parasites or to a Th17 response affecting extracellular bacteria, responses which can be promoted by selective TLR agonists. The therapeutic use of natural

Table 1. Toll-like receptors (TLRs), their natural ligands and some pharmacological modulators (from [1])
Tablica 1. Receptori slični Tollu (engl. TLRs), njihovi prirodni ligandi i neki farmakološki modulatori (iz [1])

TLR	Natural ligands	Pharmacological modulator	Clinical indication
TLR1	Bacterial diacyl lipopeptides		
TLR2	Bacterial and viral diacyl and triacyl lipopeptides, fungal zymosan, heat shock protein 60		
TLR3*	Viral signal-stranded DNA and double-stranded RNA	Poly(I:C) derivatives	
TLR4	Viral glycoproteins and bacterial lipopolysaccharide (LPS)	BCG, Monophosphoryl lipid A, Paclitaxel	Adjuvant for HBV vaccine, cancer
TLR5	Bacterial flagellin		
TLR6	Bacterial triacyl lipopeptides		
TLR7* and 8*	Viral single-stranded DNA	Imiquimod, resiquimod	Genital warts caused by human papilloma virus
TLR9*	Unmethylated cytosine-guanine dinucleotide (CpG) motifs in bacterial and viral DNA		
TLR10	Unknown		
TLR11	Protozoal protilin-like molecules		

* intracellular receptors

cytokines, such as interferons, is another means to enhance a particular type of adaptive immune response. Other soluble mediators can also facilitate activation of T regulatory (T reg) cells, which modulate the adaptive immune response. While many immunomodulatory effects of therapeutic modalities are mediated by actions on antigen-presenting cells, there is also evidence that direct effects on lymphocytes are possible. Here a brief overview is presented of some of the mechanisms involved in prophylactic and therapeutic modulation of the immune response in infections. Generally, it can be stated that the more selective the action of the immunomodulator, the more effective the outcome in terms of host defence.

Effects of exercise on host defence

The health benefits of moderate exercise are widely recognized and frequently presented in the media. These benefits, though, are not restricted to increased muscle tone and reduction of fat. Lack of physical activity can decrease life expectancy by up to 5 years, facilitate cancer development and the appearance of autoimmune diseases. This is thought to be due to the absence of stimulatory effects of moderate exercise on the immune system [2]. Active muscle tissue releases the cytokines, interleukin-6 (IL-6), IL-15 and tumour necrosis factor-α (TNFα), which are more commonly considered to be pro-inflammatory mediators [3, 4]. Acute or moderate exercise in non-athletes thus causes an increase in circulating pro-inflammatory cytokines and stimulation of innate immunity. Natural killer (NK) cell and CD4 Th cell counts increase and there is evidence that the frequency of upper respiratory tract infections is reduced [3]. My mother's advice to wrap up and get some exercise when, as a child, I had a cold has proved to be well-founded!

Since exercise is commonly taken in the open air, it is also worth noting that exposure to sunlight increases the production of vitamin D which has been shown in recent investigations to be required for effective immune responses. However, there is currently no clear evidence for reproducible enhancement of resistance to infections by vitamin D [5].

Intensive exercise and training, as opposed to moderate exercise, is associated with a decrease in IL-6 production and in NK cell counts, and a concomitant increase in the frequency of respiratory infections in athletes [3]. These individuals are candidates for prophylactic and therapeutic pharmacological immunomodulation.

IL-6 and TNFα are also adipokines, produced by adipose tissue and are modulators of lipid and glucose metabolism. Their chronic low-level production in obese individuals skews the monocyte/macrophage response towards a pro-inflammatory phenotype that promotes metabolic diseases [6]. The regulator of this balance is another adipokine, adiponectin, which inhibits the production of the pro-inflammatory IL-6 and TNFα and is present in high concentrations in lean individuals. Regular moderate exercise, associated with a healthy balanced diet can thus enhance host defence against infection and protect against the subsequent development of metabolic disease. A balanced diet should provide an adequate supply of vitamins and sufficent plant antioxidants which have a variety of beneficial actions on host defence.

Natural dietary polyphenols and resistance to infection

Many plants contain high amounts of polyphenolic compounds, such as the flavonoids, and several thousands are thought to occur in commonly used dietary plants and extracts. The flavonoids consist of six classes of compounds, the anthocyanidins, catechins or flavanols, flavones, flavonols, flavanones and isoflavones. A wide variety of these flavonoids – either as fruit juices, extracts or isolated compounds – have been extensively investigated in recent years. This is because of their potential beneficial effects on the cardiovascular system, cell growth and host defence reactions [7]. They are all antioxidants and also exert wide-ranging inhibitory effects on inflammatory cytokine production, mainly through inhibition of the transcription factor, nuclear factor κB (NFκB) [8, 9]. Among these dietary, antioxidant plant products, several have been investigated as adjuvants in the prophylaxis or treatment of infectious diseases.

Cranberry (*Vaccinium macrocarpon*) juice is a traditional remedy for the amelioration of urinary tract infections, which commonly occur in women. It has been shown to inhibit the adhesion to the urinary epithelium of *Escherichia coli* and other gram-negative urinary pathogens. The active ingredients appear to be proanthocyanidins [10]. However, clinical data provides better support for prophylaxis with cranberry juice than its therapeutic use. At least two relatively large, placebo-controlled studies have shown that regular 12-month ingestion of cranberry juice reduces the frequency of UTIs (Lynch 2004) [10]. To achieve this result, a glass of concentrated cranberry juice must be taken 2–3 times daily. For UTI prophylaxis, cranberry juice appears to be more effective than probiotics [10].

Tea (*Camellia sinensis*) has been highly regarded for its medicinal properties for centuries, and is a rich source of antioxidant polyphenols. Flavones and catechins, particularly epigallocatechin-3-gallate (EGCG) are present in appreciable quantities in green tea. Topical application of green tea polyphenols or EGCG has been shown to exert anti-inflammatory activity and immunomodulatory effects have been reported, though the mechanism(s) of ac-

tion has not been clarified [7]. EGCG has also been shown to exert in-vitro antibacterial effects on *Staphylococcus* species as well as some multi-drug resistant organisms responsible for nosocomial infections, though at relatively high concentrations [11–13]. In addition, anti-viral actions have also been observed *in vitro* [14]. An extract of tea has been reported to reduce clinical symptoms of influenza infection and to enhance γδ T lymphocyte functions in a double-blind clinical trial [15]. The benefit of tea compounds has been further confirmed in a recent five-month randomized, placebo-controlled study in 200 volunteers in which capsules of tea catechins and theanin were found to significantly reduce the incidence of flu symptoms and increase symptom-free periods [16]. γδ T cells rapidly produce inflammatory cytokines and appear to exert a more innate than adaptive function, in comparison to other T cell populations. Their stimulation could offer an explanation for the immunomodulatory properties of tea components [17].

Several components of green tea have been shown to exert beneficial effects in *Helicobacter pylori* infection [18]. EGCG inhibits colonization of the bacteria and the *H. pylori*-induced, TLR4-mediated death of gastric epithelial cells, while an acid polysaccharide from green tea inhibits adhesion of the bacteria to the tissue epithelium. Added to a combination of metronidazole and clarithromycin, EGCG was found to facilitate clinical eradication of *H. pylori* [18]. Because of their chemical instability, though, green tea constituents can only be considered for adjuvant therapy. There is also some animal experimental data suggesting that EGCG may be useful in reducing mortality during sepsis, but clinical support has not yet been provided [9].

Another polyphenol which has been intensively studied in recent years is resveratrol (*trans*-3,4′5-trihydroxystilbene), a constituent of red wine. Chilean wine was reported to have a particularly high content of resveratrol [18]. Croatian wines have not been investigated for resveratrol content, but several Serbian red wines have been reported recently to contain 0.11–1.69 mg/L of the active trans-isomer [19]. It promotes lipid and glucose metabolism and is under investigation for the treatment of diabetes. Resveratrol also appears to be directly bacteriostatic. It is a potent inhibitor of *H. pylori* growth *in vitro* and both resveratrol and red wine extracts have been shown to inhibit *H. pylori*-induced gastritis in mice [18]. There are also some clinical studies which suggest that moderate red wine consumption is associated with a reduction in active infection with *H. pylori* [18]. Animal data indicate that resveratrol prophylaxis or treatment, like EGCG, is of benefit in sepsis, but no clinical studies for this indication have been reported as yet [9].

The beneficial effects of resveratrol on infectious inflammation of the gastrointestinal tract, however, go beyond just effects on bacteria. In *Toxoplasma gondii*-induced intestinal inflammation in mice, oral administration of resveratrol (or another flavonoid curcumin) ameliorated

inflammation, reducing pro-inflammatory cytokines, neutrophil and T lymphocyte infiltration [20]. At the same time, the numbers of regulatory T lymphocytes (Treg) in intestinal mucosa increased together with increased proliferation of epithelial cells. Resveratrol increases the level and activity of sirtuin-1 (SIRT1), a mammalian NAD+ dependent deacetylase, leading to deacetylation of histones and decreased expression of certain genes, including those for several pro-inflammatory cytokines [21]. SIRT1 activation also increases the expression of Forkhead box protein-3 (Foxp3), a crucial transcription factor for Treg activation. Thus, in addition to the anti-inflammatory actions it shares with other polyphenols, resveratrol is able to exert a modulating effect on adaptive immune responses through activation of regulatory T cells. Resveratrol and another flavonoid, quercetin, also have been reported to protect monocytes from *Salmonella enterica* serovar Typhimurium-induced apoptosis and cell death [22].

Interestingly, oleuropein, a mixture of well known (e.g. oleochantal and tyrosol) and unknown biophenols, and which contributes to the bitter taste of extra-virgin olive oil, has also been shown to ameliorate *Pseudomonas aeruginosa*-induced experimental sepsis in rabbits, without exhibiting antibacterial activity *in vitro* [23]. Since circulating cytokines and inflammatory mediators were reduced, it was proposed that the oleuropein was acting directly to inhibit leukocyte function.

Taken together, the effects of dietary phenolic compounds indicate that they broadly promote non-specific host defence reactions. A diet that contains plenty of fruit or fruit juice, tea and vegetables is likely to be a suitable beneficial source of these phenolic compounds. Resveratrol is under investigation as a specific candidate drug for a variety of indications.

Dietary micronutrients essential for adequate defence against infection

There is a very extensive literature on the health benefits of dietary and supra-dietary supplements of the antioxidant vitamins C and E [7]. While vitamin C is undoubtedly a crucial component of the body's antioxidant arsenal, the ability of high doses of vitamin C to further enhance immune function remains unclear. This is due, at least partially, to its rapid clearance from the body. There is some clinical evidence that, in aged patients and athletes at risk, high dose vitamin C supplements may reduce the severity and duration of the common cold [24].

Vitamin E, however, is highly lipid soluble, is rapidly absorbed after oral ingestion and accumulates in cell membranes. Vitamin E deficiency is rare, but with aging, general antioxidant mechanisms tend to be compromised and nutrition can often be sub-optimal. In both artificially vitamin E deficient animals and in elderly human subjects,

supplementation with vitamin E has been shown to enhance humoral and cellular immunity, particularly delayed hypersensitivity responses to T cell-dependent antigens [7, 25]. The degree to which this improved immunocompetence is associated with enhanced resistance to infectious diseases is unclear, although in animal studies, vitamin E supplementation does provide protection [25]. In a randomized, double-blind, placebo-controlled study in 617 elderly subjects over 65 years of age, supplementation with vitamin E 200 IU/day for 12 months had no effect on the incidence and duration of respiratory infections as a whole [25]. However, the incidence of the common cold was significantly reduced in the supplemented subjects. These findings suggest that long-term supplementation of the elderly with moderate amounts of vitamin E may prophylactically increase resistance to mild forms of respiratory viral infections.

Another antioxidant micronutrient that is crucially important for general health is the element selenium. Dietary selenium is incorporated, as selenocysteine, into a family of selenoproteins. Among these are the enzymes glutathione peroxidase (GPX) and thioredoxin reductase. The latter catalyzes the NADPH dependent reduction of thioredoxin and is involved in cell growth and signalling, while the four GPX isoforms provide defence against oxidants and peroxidation of membrane lipids [26]. In some geographical areas, the soil and therefore crops grown in it are deficient in selenium, resulting in selenium deficiency disease. This is associated with increased peroxidative damage to tissues and thyroid dysfunction. It has been shown in animals that neutrophil and lymphocyte functions are also compromised in selenium deficiency. Autocytolytic damage occurs to phagocytes responding to pathogens with an oxidative burst and there is consequent oxidative injury to neighbouring cells [27]. Dietary or stress-induced selenium deficiency symptoms, including defective immune functions, can be reversed by recommended amounts of dietary selenium. The use of selenium supplementation at doses above 50–100 µg selenium/day in dietary adequate individuals, though, is limited by toxicity [7].

Extensive studies have been carried out on the effects of selenium deficiency on influenza virus infection in mice [28]. In inital stages of the infection, selenium deficiency results in a greater mononuclear cell inflammation in lungs, probably due to enhanced reactive species generation. Subsequently, monocyte, CD4 and CD8 T cell counts decrease, but without any change in pathogen-specific antibody production [28]. This indicates that cellular, rather than humoral immunity during viral infection is more susceptible to selenium deficiency. Clearly some clinical studies are needed on selenium supplementation in influenza infection.

Patients with human immunodeficency virus (HIV) infection have been shown repeatedly to exhibit a relative se-

lenium deficiency and there is mixed data showing increases in CD4 cell counts, with reduced hospitalization and diarrhoea in response to selenium supplementation [29]. In many critically ill patients, as in the intensive care unit, endogenous antioxidants, including selenium, rapidly become depleted necessitating some form of supplementation [30]. Recently, determination of plasma levels of the major selenium tramsport protein, selenoprotein P, has been shown to be a potential marker for selenium status in critically ill patients [31].

A well-known symptom of extreme selenium deficiency is cardiac muscle damage and in mice selenium supplementation can ameliorate cardiac damage in response to Chagas diseas, caused by *Trypanozoma cruzi* parasites [32]. This is undoubtedly due to the reversal by the selenium of the reduced defence against phagocyte-derived reactive oxygen species. Autocytolytic injury to leukocytes is also likely to be responsible for the increased susceptibility of selenium deficient mice to infection with *Listeria monocytogenes* [33]. Selenium deficiency in animals results in reduced killing and/or increased susceptibility to *Candida albicans, Salmonella tyhimurium* and *Plasmodium bergeii* [28]. However, little clinical data is available on the effect of selenium supplementation on such non-viral infections.

Zinc is an essential micronutrient, required for a wide variety of biochemical reactions including DNA synthesis and the effective functioning of the immune system and for wound healing [7]. Responses of neutrophils, monocytes, NK cells, antibody-producing B cells and the development and activation of lymphocytes, including cell-mediated killing of viruses, have all been shown to be dependent on zinc [7, 34]. Although present in all foods, it is not stored in the body, and vegetarians need a higher intake of zinc, because its absorption from plant foods is lower than from meat. Relative zinc deficiency can occur due to malnutrition and in critically ill patients. In developing countries, zinc supplements are now widely used to prevent diarrhoea in malnourished children. In the intensive care context, zinc supplementation is crucial to maintain vital functions in the critically ill patient [30].

Zinc has been shown to inhibit the replication of rhinoviruses [24]. Data on the use of zinc supplementation in the common cold, though, are somewhat contradictory and appear to be dependent on the form of zinc used. A recent Cochrane review of larger randomized, placebo-controlled trials indicates that prophylaxis with zinc in healthy young children reduces the incidence of the common cold [35]. Therapeutic treatment for up to 7 days with zinc, within 24h of the appearance of symptoms in otherwise healthy people, also reduced the duration and the severity of the common cold. It seems likely, therefore that, even in healthy people, zinc supplements may be of benefit in mild-moderate acute respiratory viral infections.

Zinc supplementation, particularly together with antibiotics, moreover, has been proposed to reduce the incidence and severity of community acquired pneumonia (CAP) in very small children [24]. A more extensive review has confirmed this finding, particularly in small children in developing countries, who are likely to be zinc deficient due to poor nutrition [36].

As a general rule, ensuring that intake of micronutrients meets the recommended daily allowances (RDA) for selenium (increasing from 15 μg/day for babies to 55 μg/day for adults) and zinc (increasing from 2 mg/day for babies to 15 mg/day for adults) will be sufficient to maintain host defence to infection in normal subjects. In poorly nourished individuals, those subjected to stress and patients who are critically ill, decreases in available micronutrients probably justify additional supplementation. Zinc, in the form of an organic ligand with amino acids, can be administered at up to 5-fold the RDA in adults without significant side-effects.

Anti-inflammatory effects of dietary fatty acids

Polyunsaturated fatty acids (PUFA) have long been known to modulate lymphocyte and monocyte function and to modify host defence in inflammatory diseases [37, 38]. Particular interest has been given to the health benefits of (n-3) fatty acids present in fish oil, in view of their protective effects on cardiovascular disease. However, despite extensive experimental studies and some limited clinical investigations, the data on the effects of (n-3) PUFA on resistance to infectious diseases are contradictory [39]. The effects are dependent on duration and timing of treatment and on the pathogen involved.

It appears that (n-3) PUFA may actually impair host resistance to *Mycobacterium tuberculosis* [39]. Recently, however, the provision of fish oil-derived omega-3 fatty acids, together with selenium supplementation has been reported to provide additional benefit to the critically ill patient with sepsis [9]. So perhaps combination of the (n-3) PUFA with antioxidants may be of benefit in some infectious conditions. An explanation for this finding may be found in the fact that (n-3) is converted by lipoxygenases to resolvins and protectins, lipids which stimulate apoptosis of leukocytes and the resolution of the acute inflammatory process [30]. Further clinical studies are needed to strengthen this possibility.

Probiotics as supportive treatment for gastrointestinal infections

Probiotics consist of live orally administered, non-pathogenic microorganisms. These include *Saccharomyces boulardii* yeast and lactic acid bacteria, such as *Lacto-*

bacillus and *Bifidobacterium* species. As for the dietary factors discussed previously, the biological effects of probiotics are relatively non-specific. Benefits observed are strain and species specific and effects associated with one species or strain cannot necessarily be extrapolated to others. Their effects are exerted by various mechanisms, including reduced intestinal pH, regulation of epithelial barrier function, altered colonization and invasion by pathogenic organisms, and direct modulation of the host immune response. Effects reported on immune function include stimulation of TLRs on epithelial cells and macrophages with the generation of chemokines and cytokines and modulation of epithelial function; induction of tolerogenic DCs and the consequent activation of regulatory T cells; as well as modulation of T helper cell responses and antibody production [40, 41]. Lactic acid bacteria promote the fermentation of dietary components to short chain fatty acids, such as butyrate. This provides an energy source for the mucosa, enhances epithelial barrier protection and exerts anti-inflammatory activities, causing a shift in cytokine profile from pro- to anti-inflammatory mediators [41]. It has been proposed that these short chain fatty acids may provide a unitary mechanism for probiotic action through modulation of epigenetic processes involved in chromosome folding and gene expression [41].

The strongest evidence for the clinical effectiveness of probiotics is found in the treatment of acute diarrhea, caused by bacteria, viruses or other factors such as drugs or radiotherapy [42]. In addition, probiotics appear to reduce the gastrointestinal effects of antibiotics in *H. pylori* eradication and prevent reoccurrence of pouchitis in inflammatory bowel disease. They may help to boost the immune response during vaccination. Other indications are under intensive investigation. Further details on the immunomodulatory effects of probiotics are provided in the accompanying article by Donatella Verbanac in this issue of the Croatian Journal of Infection.

Phytopharmaceuticals as immunomodulators

The field of plant-derived pharmaceuticals is complex and at times confusing. It is complicated by the fact that many phytopharmaceuticals are used both for their pharmacological effects and as homeopathic remedies and that some isolated dietary plant products, such as resveratrol and EGCG, are being studied as drugs. Since homeopathy is based on philosophical, rather than scientific grounds, it is not strictly within the scope of a pharmacological analysis. Traditional Chinese and Indian Ayurvedic plant medicines are based on synergistic effects of different constituents and have, therefore, been difficult to evaluate. As a result of the standardization of active components, these preparations have come under increasing evidence-based scientific scrutiny and many are proving to exhibit clear beneficial pharmacological effects. This field has been re-

viewed recently [43]. Immunomodulatory phytopharmaceuticals commonly available in the West tend to be non-specific stimulators of innate immune responses.

The squeezed sap of the upper aerial part of the purple coneflower (*Echinacea purpurea*) is widely used as a non-specific immunomodulator for mild to moderate respiratory infections, such as the common cold [7]. Among the various biologically active ingredients present in these extracts, the alkylamides are considered to represent the most active immunostimulatory principles [44]. They are effective stimulators of the phagocytosis of macrophages and inhibit their release of inflammatory cytokines, such as tumour necrosis factor-α (TNFα), apparently through an agonistic action on cannabinoid CB2 receptors [44]. Other reported actions include stimulation of NK cells, increases in circulating leukocytes and weak anti-viral activity. Isolated acid polysaccharides were recently found to ameliorate disease progress (detemined as body weight loss) in mice infected with influenza virus and to reduce the lung concentrations of interferon-γ and proinflammatory chemokines [45]. Viral titres, though, were unaffected.

When given orally within a few hours of the first signs of a common cold, the standardized sap of Echinacea appears to shorten the duration but not the intensity of the symptoms [7]. Here, once again, it is important to differentiate these effects of the squeezed sap from those of homeopathic mixtures, which are often prepared from the roots of the plant. In this respect, it is worth pointing out that in a recent in-vitro study, only the alkylamides from the aerial part of the plant were reproducibly effective inhibitors of macrophage cytokine release, not extracts of the roots [46].

Ginseng is a traditional Chinese medicine which is widely available in the West for a wide range of proposed indications. Isolation of individual components revealed that the ginsenosides show clear immunostimulant activities. They have been shown repeatedly to exhibit adjuvant activity in combination with vaccines, wth potency similar to that for aluminium hydroxide [47]. Ginsenoside Re appears to be a combined adjuvant for both Th1 and Th2-mediated humoral responses, as shown with an influenza vaccine [48]. There are as yet no clinical studies, however, which have demonstrated the relevance of this activity of ginsenosides for the therapeutic use of crude ginseng extracts [49].

The value of a variety of other phytopharmaceuticals for the treatment of upper respiratory tract infection has been reviewed [50]. Generally, the clinical effectiveness of these non-specific stimulators of innate immunity is improved by standardising the plant extracts on the basis of defined constituents with circumscribed effects on the immune system. Extracts which contain higher concentrations of these known constituents exert more reproducible and measurable effects.

Antibacterial drugs with additional immuno-modulatory properties

Most antibacterial drugs are inhibitors of bacterial protein synthesis, so it is not surprising that they also interfere with mammalian protein synthetic reactions. This leads not only to adverse effects of the drugs, but also to varied anti-inflammatory and immunomodulatory activities which enhance the therapeutic benefit of the antibiotics in bacterial infections [51]. A crucial factor in effects on host defence reactions is that many of the drugs accumulate in white blood cells after oral administration. The beta-lactams, such as amoxycillin, exhibit poor cellular accumulation, while the the cyclines and quinolones generally accumulate 2-10-fold and grepafloxacin as much as 66-fold in leukocytes [51]. The macrolide antibacterials, azithromycin in particular, are the champions at accumulation, achieving intracellular concentrations in leukocytes several hundred-fold higher than those in plasma.

Table 2.   Antibacterial drug effects on the neutrophil oxidative burst in vitro (from [51])
Tablica 2. *In vitro* utjecaj antibakterijskih lijekova na oksidativni prasak neutrofila (iz [51])

Class	Example	Effect	Possible mechanism
Ansamycins	Rifampicin	Inhibition	Inhibition of NFκB activation
Benzylpyrimidines	Trimethoprim	Inhibition	Inhibition of phosphatidate phosphohydrolase, a component of the NADPH oxidase system
Some beta-lactams	Ampicillin, Aminothiazolyl Cephalosporins	Inhibition	Oxidant scavenging, myeloperoxidase inhibition
	Cefotaxime, Cefodizime, Imipenem	Enhancement	Effects on Ca^{++}, GM-CSF release (cefodizime)
Cyclines	Minocycline, Doxocycline	Inhibition	Ca^{++} chelation, oxidant scavenging
Macrolides	Azithromycin, Clarithromycin	Inhibition	Effects on transcription factors AP-1, NFκB
		Enhancement in naive neutrophils	

Many studies have been carried out on the effects of antibacterials on the neutrophil oxidative burst *in vitro*. On the one hand, this process is an essential component of the host antimicrobial defence armamentarium. On the other hand, excessive oxidant production during phagocytosis of bacteria by neutrophils results in undesirable bystander injury to surrounding cells and tissues. Among antibacterial classes shown to exert effects on the neutrophil oxidative burst, the majority inhibit the response (Table 2). However, some individual beta-lactam drugs actually enhance the oxidative burst, mainly in association with other stimulatory effects on neutrophil function. This would be expected to contribute towards enhanced bacterial killing though it is unclear whether increased surrounding tissue injury is involved. The macrolides, including azithromycin, although inhibiting the neutrophil oxidative burst to pathogens and particulate stimuli *in vitro*, enhance the ex-vivo neutrophil oxidative response when administered to healthy animals and humans [52, 53]. This appears to be due to a biphasic response by which the drug initially stimulates naive neutrophils (i.e. before they enter an infected/inflamed site) but then downregulates the response following stimulation by the pathogen, thereby reducing bystander tissue injury.

The cephalosporin, cefodizime, is a specific exception to the other compounds in its class. Not only does it stimulate neutrophil phagocytic and bactericidal capacities, it also increases the formation of granulocyte-macrophage colony stimulating factor (GM-CSF), required for neutrophil survival, as well as the production of inflammatory cytokines *in vivo*. The mechanism remains unclear. In immunocompromised animals, cefodizime was effective against several drug resistant strains of *Plasmodium berghei*, *Candida albicans* and *Toxoplasma gondii* and promoted neutrophil phagocytosis in immunocompromised patients (e.g. cancer, hemodialysis, old age) [54]. However, there is no clear evidence that this immunomodulatory activity provides an advantage for cefozidime over other cephalosporins in clinical practice [55].

This is not the case with several other classes of immunomodulatory antibacterials, including the cyclines and macrolides. In community acquired pneumonia (CAP), macrolides show distinct advantages over other antibiotics in reducing mortality and length of hospitalization, which is attributed to their immunomodulatory and anti-inflammatory effects [56]. In ventilator-associated pneumonia (VAP) and sepsis in 200 patients, early initiation of treatment with clarithromycin for 3 days significantly shortened the time to resolution of VAP and even the relative risk of death by septic shock and multiple organ failure in comparison to placebo [57]. These and many other beneficial effects of macrolides in infectious and inflammatory diseases are attributable to their immunomodulatory actions, in addition to their direct antibacterial actions [58, 59]. The list of these actions is long and includes inhibition of neutrophil activation and promotion of their clearance by apoptosis; inhibition of proinflammatory cytokine release from a variety of different immune cells and the modulation of macrophage differentiation; inhibition of fibroblast proliferation and angiogenesis and adhesion in endothelial cells; inhibition of mucus secretion and mediator production by airway epithelial cells [58]. Based on their immunomodulatory properties and some limited clinical case studies, it has been proposed that macrolides may be used to control the cytokine storm in influenza viral infections [60]. In addition, azithromycin inhibits quorum sensing and biofilm production by *Plasmodium aeruginosa*, which contributes to its therapeutic benefit in cystic fibrosis [61, 62]. A more detailed discussion of immunomodulatory macrolides is given by Vesna Erakovic Haber in an accompanying article in this issue of the Croatian Journal of Infection.

Tetracyline antibacterials, like macrolides, also exhibit pronounced anti-inflammatory and immunomodulatory actions [51]. Although the use of tetracyclines as antibiotics has declined because of increasing bacterial resistance, minocycline and doxycycline in particular are still used as anti-inflammatory/immunomodulatory agents in the treatment of a number of non-infective diseases. These include rosacea and scleroderma, periodontal disease and rheumatoid arthritis [63]. This clinical activity is thought to be due mainly to inhibitory effects on matrix metalloproteinases (MMPs), which are responsible for tissue breakdown and angiogenesis in a variety of chronic inflammatory diseases, as well as cancer [63, 64]. Doxycycline is the most active tetracycline in this respect. It inhibits MMP-1, -2, -7, -8, -9, -12, and -13 directly by binding to zinc at the active site of the enzyme, but also inhibits MMP gene expression and the production of the enzymes [64]. In addition, the phenolic tetracycline structure is responsible for reactive oxygen scavenging properties and the drugs as a class exert anti-apoptotic activity and reduce neutrophil migration and adherence [51, 65]. Both minocycline and doxycycline are undergoing clinical trials for a wide range of different indications, including neurodegenerative disease [65] and a variety of tetracycline analogues are currently under investigation as novel anti-inflammatory agents [51, 64].

The anti-MMP, antioxidant and anti-inflammatory activities of tetracyclines undoubtedly complement their efficacy as antimicrobial drugs in the treatment of severe periodontal disease, particularly that involving *Aggregatibacter actinomycetemcomitans* [66]. It is suggested that they are most effective in young people at risk, in patients with systemic inflammatory disorders and those with acute periodontal disease as adjuvant therapy to root surface debridement. In acne, the antimicrobial activity of topical tetracyclines is considered to be complemented by their antioxidant activity, leading to reduced erythema [63].

Many other antibacterials have been shown to modulate inflammatory cytokine production or to exert immunomodulatory effects in animal studies. However, in comparison to macrolides and tetracyclines, few of the immunomodulatory effects of other antibacterials have been shown to be relevant to their clinical activities or led to their use in non-infectious indications [51].

Among antibacterials which do possess beneficial immunomodulatory and other effects, these are predominantly serendipitous and represent non-selective effects which are secondary to their primary activities as antibacterial drugs. The drug structures were not optimized specifically for these immunomodulatory effects, although both novel macrolide and tetracycline compounds are now being investigated as targeted immunomodulatory and anti-inflammatory drugs.

Specific receptor-mediated immunomodulators in anti-infective therapy

The primary characteristic of immunomodulatory agents, developed and used primarily for infectious indications, is the specificity of their actions on cellular targets. The classic immunomodulatory agents are preparations of immunoglobulins (Igs) prepared from human plasma for intravenous administration (IVIG). They are used for the treatment of autoimmunity, for maintenance therapy in genetic Ig deficiency states and for passive immunization against infections. Although synthesized in response to highly specific antigens, the specificity of the action of pooled, polyvalent IVIG preparations, which bind to a variety of epitopes, is not immediately apparent. This specificity is provided by the binding of Igs to Fc receptors. In autoimmunity, IVIG probably act by complement scavenging and inhibition of binding of autoantigenic immune complexes to Fc receptors for Ig on mononuclear phagocytes [67].

In infectious diseases, their use in passive immunisation is predominantly based on neutralisation of the infectious agent and clearance of the resulting immune complexes by binding to Fc receptors on phagocytes. In recent years, hyperimmune plasma from volunteers immunised with approved vaccines against diseases such as hepatitis B, varicella zoster, tetanus toxoid or rabies has been used increasingly as a source of pathogen-specific, high-titre therapeutic Ig [68]. This type of pathogen-specific plasma is used predominantly when the patient is suspected of being infected and passive immunization with IVIG is given to allow time for specific active immunization.

A variety of different immunomodulators are used specifically for the prevention or therapy of infectious diseases. These include recombinant cytokine drugs, as well as synthetic immunomodulators [69]. Again, their clinical benefit in infectious diseases is closely related to their spe-

cific actions on discrete cellular receptors. It is beyond the scope of this article to review all these various approaches, but two of the most important should be mentioned as examples.

Recombinant human cytokine therapies are best illustrated by the interferons. The recombinant human type I interferons are mainstays for the therapy of chronic hepatitis infection [70, 71]. Though acting on specific cellular receptors, the downstream effects of the interferons are remarkably varied. Interferons (IFNs) α and β differ in their protein structures but act on the same receptor, IFNαR. The activation of the IFNαR receptor leads to the induction and expression of more than 300 interferon-stimulated genes (ISGs) which are involved in host defence against viral infection, including ISG15 which plays a pivotal role in protection against HCV [72, 73]. These host defence responses include enzymes which promote the degradation of viral RNA and inhibition of viral protein synthesis in virally-infected hepatocytes. In addition, binding to the IFNαR on antigen-presenting cells (APCs) leads to an increased cellular activation state. This includes enhanced expression of cell surface molecules, including Fc receptors for Ig and the major histocompatibility complex (MHC) class I and II molecules, with which specific (e.g. hepatitis viral) antigen must be associated in order to induce T cell activation and cytotoxicity to infected cells [69]. Cytokine generation by the APCs is also enhanced. Together these actions of type I interferons result in stimulation of the adaptive immune response to hepatitis viral antigens. In hepatitis patients showing a good clinical response to interferon therapy, usually for 24–48 weeks, a shift from a Th2 to a mainly Th1 immune response is frequently observed.

In clinical practice, type I interferons are used predominantly as conjugates with polyethylene glycol (PEG), which enhances chemical stability, decreases protein degradation and reduces immunogenicity. The plasma half-life of PEGylated IFNs is considerably prolonged, in comparison to the native protein, with an associated improvement in efficacy [69]. While PEGylated IFN-β is mainly used in the treatment of multiple sclerosis, herpes zoster and varicella in immunosuppressed patients, PEGylated IFN-α2 is used for therapy of both HBV and HCV infection. The standard therapy for HCV is PEGylated IFN-α2 in combination with the antiviral drug ribavirin [71]. With these therapies, a sustained reduction of viral DNA can be observed in 40–60 % of patients. In genotype 2 HCV-infected patients, the standard IFNα plus ribavirin therapy is effective in 80 % of patients [74]. The disadvantage is that the treatment leads to anaemia and blood cytopenias.

Recently, it has been shown that the gene IL28B, which regulates the expression of a third class of interferon, IFNλ3, plays a crucial role in natural defence against HCV, inducing the activation of a different set of ISGs to those

that are generated by IFNαR binding. The expression of IL28B gene-dependent products appears to be closely associated with the treatment success of PEGylated IFNα and ribavirin in HCV infection and may be an effective means for monitoring therapy [75]. As a corollary, induction of ISG15 by HCV prior to therapy appears to be a predictive factor for resistance to IFNα therapy [73].

The recombinant human colony stimulating factors (CSFs), filgrastim (G-CSF) and sargramostim (GM-CSF), provide further examples of highly specific cytokine therapy. They act on specific G-CSF and G-CSF receptors to stimulate the differentiation and growth of granulocytes, and are used to reverse neutropenia and reduce infections in patients submitted to radiotherapy or chemotherapy [69]. GM-CSF, which in addition stimulates the differentiation and growth of monocytes/macrophages, has also been proposed to be of benefit in patients with sepsis who have compromised immune function. However, a recent meta-analysis of several small-scale trials concludes that the evidence is currently not convincing [76].

In this respect, it is worth mentioning the findings of a recent meta-analysis of 18/502 clinical studies which met the criteria for investigation of the effects of potentially immunomodulating interventions in comparison to placebo on infection, multiple organ failure (MOF) and mortality in trauma patients [77]. The interventions included glucan (a component of the inner cell wall of *Saccharomyces cerevisiae* which stimulates bone marrow proliferation), dextran, IFN-γ, immunoglobulins, leukocyte-reduced blood, prostaglandin E, antioxidants and antithrombin III. (The effects of monoclonal antibodies against the adhesion molecule CD18 were also included, but these are purely inhibitory on defence reactions rather than being immunomodulatory). Significant changes in infection, MOF and mortality rates were only observed in the studies testing IFN-γ, immunoglobulin, and glucan. These findings support the idea that agents with specific actions on the immune system are more effective than non-selective immunomodulators in reducing infection.

The final group of immunomodulators used in infectious diseases are the synthetic drugs, illustrated by the TLR receptor agonists. In contrast to older microbial products, probiotics and several phytopharmaceuticals, which exert non-specific effects on PRRs, including TLRs, the synthetic agonists, imiquimod and resiquimod, are highly selective agonists at intracellular TLR7 and TLR8 receptors for single-stranded viral DNA. The stimulation of TLR7/8 in APCs causes the release of type I IFNs and interleukin-12 (IL-12) which act on T lymphocytes to bias the activation response towards Th1 cells which are needed to mount an effective cytotoxic defence against viral infection [69]. As a consequence, imiquimod and resiquimod are effective by topical application in eliminating genital warts caused by human papilloma virus (HPV) [78]. Other selective TLR agonists, such as the TLR4 ago-

nist monophosphoryl lipid A (in HBV vaccine), are used as adjuvants in vaccines to bias the immunization process towards a selective T cell response to viral or bacterial antigens. Specific agonists at other TLRs are also under investigation in order to develop immunomodulators which lack the adverse effects of non-selective immune stimulation.

Conclusions

The clinical efficacy of immunomodulatory approaches to the treatment of infections is largely a case of specificity and potency. The most effective is the most specific – prophylactic immunization with a defined antigen. This can be made even more selective by including an adjuvant which is a specific agonist at a particular TLR. A healthy lifestyle, incorporating regular moderate exercise, and a diet containing sufficient micronutrients and plant polyphenols is also a rational, non-specific way of maintaining adequate host defence against infection, particularly of the upper airways and urinary tract. Probiotics provide non-specific support to gastrointestinal immunity. Phytotherapeutics offer an additional approach to general upregulation of innate immune responses but are likely to be superceded in the future by more selective agents for stimulation of antigen-presenting cell activity.

A number of antibiotics combine direct antibacterial actions with immunomodulatory or anti-inflammatory effects. These not only further promote host defence reactions but also contribute towards protection of surrounding tissues from injury by phagocytes activated by micro-organisms. At least for some macrolides, these additional immunomodulatory activities provide clinical benefit even in the presence of increased bacterial resistance. This appears to be the case in viral infections associated with a cytokine storm.

The value in infections of immunomodulators which target specific receptors rather than broadly promoting host defence is best illustrated by recombinant human cytokines. Type I interferons are widely effective in the treatment of viral infections and hepatitis in particular. But even though they act on a single receptor, the plethora of interferon stimulated genes (ISGs) means that adverse effects of interferon therapy are common. Agonists at other cytokine receptors, such as CSFs, as well as selective TLR agonists, are likely to become the most promising immunomodulatory approaches to infectious diseases in the future.

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